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Fine Mapping of an Apparently Targeted Latent Human Herpesvirus Type 6 Integration Site in Chromosome Band 17p13.3

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An unusually high level of latent HHV-6 infection has been documented in the peripheral blood and/or bone marrow cells of a small group of patients with predominantly malignant lymphoid disorders, and in at least one healthy individual. We have shown previously in peripheral blood mononuclear cells (PBMCs) of three patients, two with a history of lymphoma and one with multiple sclerosis, a specific target site for latent integration of the full-length HHV-6 viral genome on the distal short arm of chromosome 17, in band p13.3. Fluorescence in situ hybridization (FISH) procedures were used to map more precisely the location of the viral integration site in one of those patients, relative to two known oncogenes mapped previously, namely *CRK,* and the more telomeric *ABR* oncogene. It is shown that the HHV-6 integration site is located at least 1,000 kb telomeric of *ABR,* and is very likely to map close to or within the telomeric sequences of 17p. This finding is significant given that human telomeric-like repeats flank the terminal ends of the HHV-6 genome. Cytogenetic studies showed evidence of karyotype instability in the peripheral blood cells infected latently. ***J. Med. Virol. 58:69–75, 1999.***

KEY WORDS: HHV-6; nonrandom integration site; chromosome band 17p13.3; lymphoproliferative; FISH; telomere

INTRODUCTION

Human herpesvirus type 6 (HHV-6) was isolated originally from peripheral blood leukocytes of six patients with various lymphoproliferative disorders, including B-cell and T-cell lymphomas. Two of the patients were infected with the human immunodeficiency virus type 1 (HIV-1) [Salahuddin et al., 1986]. A betaherpesvirus, HHV-6 shows similarities to cytomegalovirus, and is about 160 kb in size [Gompels et al., 1995]. Although named initially human B-lymphotropic virus (HBLV), HHV-6 is in fact tropic preferentially for CD4+ T lymphocytes [Lusso et al., 1988; Takahashi et al., 1989], but can also replicate in vitro in a broad range of other cell types (reviewed by Levy, 1997). HHV-6 is well recognized as the etiologic agent for exanthem subitum (roseola) [Yamanishi et al., 1988], and is a major cause of febrile illness in young children [Pruksananonda et al., 1992; Hall et al., 1994]. HHV-6 infection of adults may cause hepatitis, meningoencephalitis, and a mononucleosis-like syndrome, and there is now also evidence of an association with multiple sclerosis [Irving and Cunningham, 1990; Steeper et al., 1990; Challoner et al., 1995; Soldan et al., 1997].

DNA restriction enzyme site and serological analyses have separated HHV-6 isolates into two subgroups, A and B [Schirmer et al., 1991]. Variant A isolates include those infecting adult patients with acquired immunodeficiency syndrome (AIDS) or lymphoproliferative disorders, have similarities to the prototype GS and U1102 strains [Salahuddin et al., 1986; Downing et al., 1987], and exhibit low prevalence in the general population. Variant B isolates exhibit a high prevalence in the population, include most isolates from children with exanthum subitum [Dewhurst et al., 1993], and in adults, the prototype Z-29 strain and other Z-29-like strains from patients with AIDS or lymphoid neoplasias [Lopez et al., 1988; Luppi et al., 1993a].

Serological studies indicate that most people become infected with HHV-6 by the age of 1–2 years [Okuno et al., 1989; Levy et al., 1990]. Following primary infection, HHV-6 genomes persist often at extremely low

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copy number in latent form, or because of chronic residual infection [Pietroboni et al., 1988; Jarrett et al., 1990; Levy et al., 1990]. Latent virus may reactivate when patients are immunocompromised, as has been reported for a number of transplant recipients and HIV-infected subjects [Singh and Carrigan, 1996].

Previous studies have identified a small group of patients with predominantly malignant lymphoid disorders who show an unusually high level of latent HHV-6 infection in their peripheral blood and/or bone marrow cells [Jarrett et al., 1988; Josephs et al., 1988; Torelli et al., 1995]. A persistently high viral load has also been detected in the peripheral blood of one case of multiple sclerosis [Torelli et al., 1995], and in one healthy individual [Clark et al., 1996]. Molecular studies have also shown that the HHV-6 variant B genome was integrated, sometimes in its entirety, into the genome of circulating peripheral blood mononuclear cells (PBMCs) in three of these cases (1 HD, 1 NHL, 1 MS) [Luppi et al., 1993b]. Integration was apparently nonrandom in that, for all three cases, fluorescent in situ hybridization (FISH) studies revealed a high proportion of cells with HHV-6 probe-specific signals on the short arm of one chromosome 17, in band p13.3 [Torelli et al., 1995].

Using two-colour interphase FISH procedures, the HHV-6 genome integration site is shown in the PBMCs of one of these patients and is physically separate and distal to the two oncogenes *ABR* and *CRK,* and likely to reside close to, if not within, the telomeric region of 17p.

MATERIALS AND METHODS

Patient History

The clinical history of the patient, diagnosed with B-cell non-Hodgkin's lymphoma (NHL), has been reported elsewhere (case 7, Torelli et al., 1995).

Cell Preparations and Chromosome Analysis

Metaphase spreads were prepared from an phitohemagglutinin (PHA)-stimulated culture of PBMCs sampled from the patient during clinical remission of non-Hodgkin lymphoma (NHL), using procedures described previously [Torelli et al., 1995]. Preparations were analysed after G-banding, and chromosome aberrations described using accepted nomenclature [Mitelman, 1995]. For some interphase FISH experiments, PBMCs had been harvested directly after Ficoll separation (Sigma, St. Louis, MO), without PHA stimulation, cell culture, or colcemid treatment, using standard hypotonic and fixation procedures.

DNA Probes

Probe pZVB70 is a HHV-6-specific, 22.5 kb Bam H1 fragment subcloned into the plasmid vector Bluescript [Kishi et al., 1988]. The genomic *CRK* probe #3 and the pooled *ABR*-specific probes 2-1, 6-1, and 8-1 have been described elsewhere [Morris et al., 1995].

Fluorescent In Situ Hybridization (FISH)

FISH methods, including probe ordering and distance measurements in interphase cells, were essentially as described [Trask et al., 1989, 1991; Senger et al., 1993; Morris et al., 1995], with some modifications. Briefly, metaphase or interphase cells spread on slides were treated with proteinase K or pepsin, paraformaldehyde fixed, and chromosomal DNA was denatured by immersion for 2–3 min at 70°C in a solution of 70% formamide/2 × SSC, pH 7.0 before dehydration in an ice-cold ethanol gradient. Probes were random prime labelled or nick-translated with biotin (Bethesda Research Laboratories, Gaithersburg, MD) or digoxygenin (Boehringer Mannheim, GmbH, Germany) and were hybridized at a final concentration of 50–100 ng/μl in a 10-μl mix containing 50% formamide, 2 × SSC, 10% dextran sulfate, and 500 ng/μl herring sperm DNA. Ten micrograms of human Cot 1 DNA (1 μg/μl) (BRL) were added to suppress repeat sequences in the probes. Probes in hybridization mix were denatured at 75°C for 5 min allowed to preanneal for 60 min at 37°C, then immediately applied, under coverslips, to the denatured metaphase or interphase slide preparations. Slides were incubated overnight at 37°C in a saturated humid chamber, then excess probe was removed by washing three times, 2 min each, in 50% formamide/2 × SSC, pH 7.0, at 45°C, followed by three 2-min washes in 2 × SSC, pH 7.0 at 45°C, and a final 15-min stringent wash in 0.1 × SSC at 60°C.

Immunological Detection

All immunological detection steps were carried out under coverslips, and in a humidified chamber at 37°C. For biotinylated probe detection, slides were blocked for 30 min in blocking reagent-B (BR-B: 3% w/v bovine serum albumin (BSA)/2 × SSC) at 37°C, then incubated in 5 μg/ml fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories, Burlingame, CA) in BR-B with added 0.1% v/v Tween-20 (Sigma) (BR-BT) for 45 min at 37°C. Probe signal was amplified with biotinylated anti-avidin (Vector Laboratories; 5 μg/ml in BR-BT) and a further layer of FITC-avidin. In some cases, a second amplification was undertaken. Each detection step was followed by three 5-min washes in 2 × SSC/0.1% Tween-20 at 43°C. For digoxygenin labelled probes, slides were blocked for 30 min in blocking reagent-D [BR-D: 0.5% w/v blocking reagent (Boehringer Manheim)/100 mM Tris.Cl, pH 7.5/150 mM NaCl], at 37°C, then incubated in sheep anti-digoxygenin (Boehringer Manheim, final concentration 4 μg/ml in BR-D), FITC-conjugated rabbit anti-sheep Ig(H + L) (Vector Laboratories, 30 μg/ml in BR-D), and a final layer of FITC-conjugated goat anti-rabbit Ig(H + L) (Vector Laboratories; 30 μg/ml in BR-D). In this case, each detection step was followed by three 5-min washes in 100 mM Tris.Cl, pH 7.5/150 mM NaCl/0.1% Tween 20 solution at 43°C. For mixed probe experiments, Texas-

Red labelled avidin (Vector laboratories; 5 μg/ml in BR-BT) was used in place of FITC-avidin to detect biotinylated probes. When digoxygenin- and biotin-labelled probes were applied to the same slide, preparations were incubated sequentially in digoxygenin detection reagents, followed by biotin detection reagents, before mounting. Although this procedure took considerably longer, we found that troublesome cross-reaction between the FITC (digoxygenin) and Texas Red (biotin) probe sites was reduced compared with other protocols, which recommend combining biotin and digoxygenin detection reagents at some steps (unpublished data).

Digital Image Processing and Analysis

After final washes, slides were mounted in glycerol containing the antifade agent *p*-phenylene-diamine dihydrochloride (Sigma, 1 mg/ml) and 0.25 μg/ml DAPI as counterstain for G-band visualization. Metaphase or interphase cells were analyzed under a Leitz Aristoplan microscope with fluorescence capabilities and images were transferred for processing to a Power Macintosh computer via a Photometrics cooled CCD camera. For two-colour interphase probe distance estimations, FITC and Texas Red images were captured separately, then overlaid on a final composite image before applying software measurement tools. Interphase cells were selected for probe ordering information or distance measurements only if the *CRK* and *ABR* probes co-hybridized with the HHV-6 probe showed clear, single, and distinct fluorescent signals representing the homologous chromosomal sites in G1 phase.

RESULTS

FISH Detection of HHV-6 Genome Integration Site on Chromosome 17p13

Consistent with studies reported previously on this NHL patient, biotinylated- or digoxygenin-labelled HHV-6 probe pZVB70 hybridized specifically to the distal short arm of one chromosome 17, band p13, in 108 of a combined total of 186 (58%) metaphase cells scored from the PHA-stimulated PBMC culture [Torelli et al., 1995, and results not shown]. Further analysis by two different observers of nonstimulated interphase PBMCs showed a single discrete signal in 45% and 52%, respectively, of 100 cells scored. This finding verifies indications from previous pulsed-field gel electrophoresis analyses that the HHV-6 genome has integrated at a single chromosomal site in a high proportion of this patient's PBMCs [Luppi et al., 1993b]. Both observers in the present study detected two well-separated fluorescent signals in 16% of interphase cells. Twelve percent (23/186) of metaphase cells analysed also showed signals on 17p and one or more other chromosomes, but there was no evidence of a second specific hybridization site elsewhere in the genome. No metaphase cell showed hybridization of pZVB70 to both chromosome 17p homologues.

Interphase FISH: Relative Ordering of *CRK, ABR,* and HHV-6

To determine the relative orientation of *ABR, CRK,* and the HHV-6 integration site, genomic probes for the three different regions were labelled with biotin or digoxygenin, and hybridized in two different combinations to the PHA-stimulated metaphase cell preparation. First, biotinylated *ABR* and *CRK* probes (detected with Texas Red) were hybridized in combination with a digoxygenin-labelled HHV-6 probe (detected with FITC). Ninety-three percent of cells analysed (37/42) showed the signal order red-red-green on the HHV-6 integrated chromosome, indicating that the viral site mapped some distance either telomeric of *ABR* or centromeric to *CRK* (Fig. 1a,b). For the second experiment, digoxygenin labelled *ABR* and HHV-6 probes were hybridized in combination with a biotinylated *CRK* probe. In this case, 74% of informative cells scored (52/70) showed the order green-green-red, indicating that the CRK gene maps proximal or distal of ABR and HHV-6 (Fig. 1c,d). From these results, and the known orientation of CRK and ABR on 17p [Morris et al., 1995], we determined the order (centromere) - CRK - ABR - HHV-6 integration site - (telomere).

Interphase FISH: Physical Distance Estimates

The measured distance between fluorescent signals after combined hybridization of two or more probes to nondividing interphase cells correlated accurately and linearly with physical kilobase distance up to 500 kb, and provided a useful and direct means to estimate the physical distance that separates two closely linked probes [Trask et al., 1989, 1991; Senger et al., 1993]. Distances between the signals in informative cells after hybridization were measured with the two- and three-probe combinations of biotinylated or digoxygenylated *ABR, CRK,* and HHV-6 probes (Table I). When hybridized in paired combinations, the distance separating *CRK* and the HHV-6 integration site (1.30 μm) was marginally greater than that separating *ABR* and HHV-6 (1.23 μm), but the difference was not significant when the standard error of the mean (SEM) was taken into account (Table I). In the triple probe experiment, the measured distance separating *CRK* and HHV-6 was considerably greater than that separating *ABR* and HHV-6 (1.99 μm vs. 1.46 μm) (Table I). A combination of chromatin conformation and the more rigorous selection process implicit in visualising three distinct signals along the chromosome compared with two signals may account for the observed differences between identical probe pairs shown in Table I. We consider that the data from the triple probe experiments reflects most accurately the true relative physical distances separating the HHV-6, *ABR,* and *CRK* loci.

When converted to kilobase pair estimates, based on accumulated data of Trask et al. [1989, 1991] and Senger et al. [1993], the approximately 450-kb physical distance separating *CRK* and *ABR* is consistent with

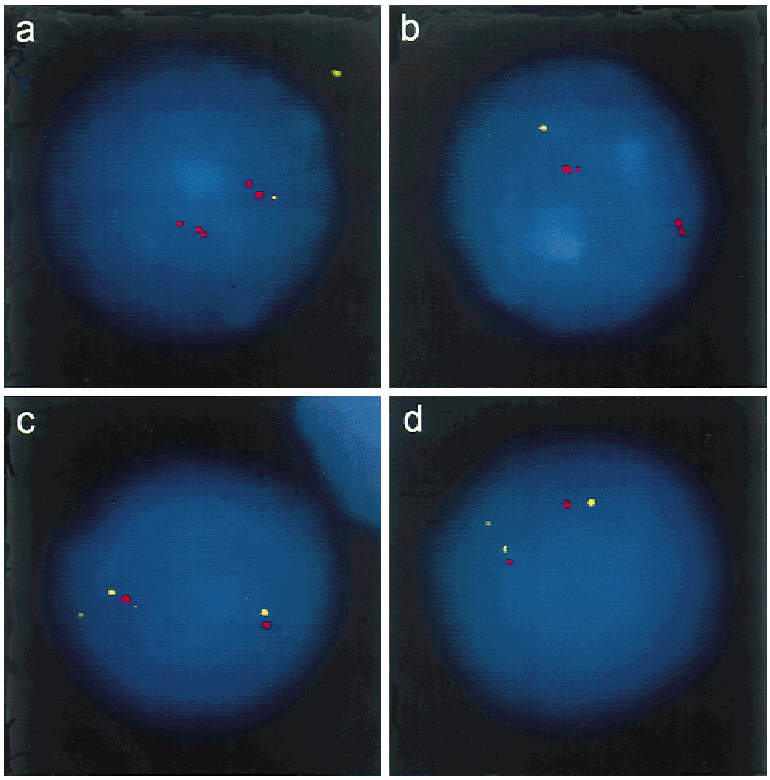

Fig. 1. Examples of phitohemagglutinin (PHA)-stimulated peripheral blood mononuclear interphase cells of the NHL patient (in remission of disease) showing most frequent signal orientation after dual colour fluorescent in situ hybridization (FISH) hybridization of HHV-6, *ABR,* and *CRK* specific probes in triple probe combinations: **(a,b)** ABR (red), CRK (red), HHV-6 (green), and **(c,d)** CRK (red), ABR (green), and HHV-6 (green).

TABLE I. Physical Distances Separating *CRK, ABR,* and HHV-6 Integration Site in Interphase Cells

Inter-probe distance measured	Paired or triple probe hybridization[a]	No. of measurements	Mean measured distance (μm ± SEM)	Estimated genomic distance (kb)[b]
HHV-6 → CRK	Paired	37	1.30 ± 0.09	⩾1 Mb
HHV-6 → CRK	Triple	25	1.99 ± 0.13	> 1 Mb
HHV-6 → ABR	Paired	40	1.23 ± 0.08	⩾1 Mb
HHV-6 → ABR	Triple	24	1.46 ± 0.09	> 1 Mb
CRK → ABR	Triple	48	0.64 ± 0.04	~ 450 kb

[a]Distance measurements were taken after combined hybridization of two probes (paired - HHV-6 + either *CRK* or *ABR*) or three probes (triple - HHV-6, *CRK* and *ABR*). Refer to Methods/Results for further details.
[b]Derived from mean measured distances and based on data from Trask et al. [1989, 1991] and Senger et al. [1993].

our previous estimate using similar procedures [Morris et al., 1995]. The distance separating these two oncogenes is distinctly shorter than the distance that separates *ABR* and the more telomeric HHV-6 integration site, an estimated distance of more than 1 megabase (Mb) (Table I). This result places the HHV-6 integration site very close to, if not within, the telomeric region of the short arm of chromosome 17.

Karyotype Studies

Standard cytogenetic analysis by one observer revealed the following abnormal karyotypes in 3 of 26 PHA-stimulated GTG-banded PBMC metaphase cells examined: 46,XX,t(5;13)(q12;q34)[1]/47,X,–X,–6,–8,–22,–22,dic(1;11)(q11;q25),del(4q),del(9p),del(10q),t(21;?)(q22;?),+6mar[1]/52,XX,–16,+19,+20+?22,17p+,+4mar[1]. The remaining 23 cells showed a normal female karyotype. Blind analysis of the same preparation by a second observer revealed the following abnormal karyotypes in 7 of 33 metaphase cells: 44,XX,del(4p),dic(2;12)(q21;q24),dic(3;22)(p11;q13)[1]/45,XX,–1,–19,+mar[1]/45,X,dic(X;6)(pter;q21)[1]/45,X,dic(X;16)(p22.1;q12 or p11;qter)[1]/45,X,–X,–5,t(3;7)(p11;q21.2)+mar[1]/46,XX,tas(2;7)(q37;p22)[1]/52,XX,–16,+19,+20,+?22,17p+,+4mar[1].

DISCUSSION

The patient studied presented with a B-cell lymphoma, and detection of the latently integrated virus in noninvolved PHA-stimulated T cells argues against the integration process per se as a direct cause of lymphoid neoplasia. This conclusion is supported by the detection of HHV-6 DNA on chromosome 17p in the PBMCs of a patient with multiple sclerosis [Luppi et al., 1993b; Torelli et al., 1995]. Nonetheless, the propensity, albeit rare, for targeted integration of HHV-6 into haematopoietic cells of patients with lymphoproliferative and immune diseases suggests that the virus may be linked to the etiology of these diseases in some way. Our mapping of HHV-6 places it telomeric to the candidate oncogenes *ABR* and *CRK* in chromosome band 17p13.3, and therefore distant to the well-characterised and more centromeric tumour suppressor gene *TP53* in band 17p13.1. Physical disruption by HHV-6 insertion into any of these loci is therefore an unlikely cause of the patient's disorder. Consistent with this finding, molecular studies of the patient's PBMCs showed a germline configuration and normal expression of *TP53* (Luppi et al., unpublished observations).

Viral integration may destabilise telomeres and other chromosomal regions, making them susceptible to recombination [Rogler et al., 1985; Sandros and Stenman, 1990; Kao et al., 1993; van der Drift et al., 1994; Wilke et al., 1996; Jox et al., 1997; Livezey and Simon, 1997; Lopez-Borges et al., 1998]. The low frequency of nonclonal chromosome aberrations we observed in PHA-stimulated cells may therefore be related to the integrated virus. The HHV-6 integration site mapped at least 1 Mb distal to the *ABR* locus, placing it close to, if not within, the telomeric region of 17p. One metaphase cell showed additional unidentified material on 17p (17p+), but four cells showed rearrangements involving terminal regions of different chromosomes [Xp, 2q, 7p, 22q, and depending on breakpoint interpretation of the t(X;16) rearrangement observed in one metaphase, either Xp or 16q]. Telomeric association of human chromosomes has been described in cell lines transformed with SV-40, Epstein-Barr virus (EBV), or human papilloma viral genes, and also in many different kinds of malignancy, including leukemia and lymphoproliferative disorders [Shippey et al., 1990; Sreekantaiah et al., 1992; Tarkkanen et al., 1993; Temperani et al., 1995; Wan et al., 1997; Sawyer et al., 1998; and refs therein]. In many of the leukaemia cases the telomeric associations were nonrandom. Leukaemic cells of one patient showed involvement of the telomeric region of chromosome 17p [Saltman et al., 1989], and it is noteworthy that clonal rearrangements involving breakage and rejoining of sites on 17p were present in three other patients [Fitzgerald and Morris, 1984; Crossen et al., 1993; Howell et al., 1993]. It would be of interest to determine if latent integration of HHV-6 featured in the leukaemic cells of any of the latter patients. We recognise that it is possible that the chromosome aberrations observed in our patient were induced by chemotherapy and persisted in long-lived T cells. However, the patient received minimal therapy, only four of the six standard courses of chemotherapy (CHOP scheme), and no radiotherapy before she entered a complete clinical remission, which still persists after 7 years.

HHV-6 has demonstrated oncogenic potential in vitro [Razzaque, 1990; Kashanchi et al., 1997], and accumulating evidence from in vivo studies suggests that this herpesvirus may be involved in a wider spectrum of human cancers than previously anticipated. The apparently nonrandom HHV-6 integration site we have identified in band 17p13.3 maps to a region that shows extensive loss of heterozygosity (LOH) in a variety of solid tumours, and in which a yet-to-be-identified tumour suppressor gene may be located. For example, LOH of 17p13.3 occurs frequently in uterine cervical carcinomas [Kersemaekers et al., 1998], ovarian cancer [Phillips et al., 1996], breast tumours [Stack et al., 1995], malignant astrocytomas [Chattopadhyay et al., 1997], and in medulloblastomas [McDonald et al., 1994]. HHV-6 DNA has been identified in a small proportion of some of these tumour types, including human papilloma virus-positive cervical cancers and neuroglial tumors [Chen et al., 1994; Luppi et al., 1995]. The possibility that LOH of 17p and viral integration are related should be investigated.

Polymerase chain reaction studies have shown that a high percentage of the normal human population carries a very low copy number of HHV-6 DNA sequences in peripheral blood cells. It will be important to determine if this viral DNA is episomal, integrating specifically at the same site on 17p, or integrating randomly at other sites, in those rare infected cells. Future work must also aim to understand the molecular basis for the apparently preferred HHV-6 integration site on 17p in lymphoid disorders. Of particular interest will be to establish if, as the present findings suggest, the human telomeric-like repeats that are known to flank both ends of the HHV-6 genome [Thomson et al., 1994; Gompels et al., 1995] are relevant to the mode by which this virus integrates into human chromosomal DNA. The telomere of the short arm of chromosome 17 is shorter, on average, than that of any other human chromosome, and may therefore be vulnerable to LOH or to rearrangements involving other chromosomal regions [Martens et al., 1998]. On the basis of our present findings, it is tempting to speculate that the shorter telomere of 17p may also be relevant to this region's propensity to integrate viral DNA. We note that a recent report described insertion of the HHV-6 genome into the distal end of the long arm of chromosome 1, band q44 in PBMCs of a patient with acute lymphoblastic leukaemia, an insertion site again suggestive of telomere involvement [Daibata et al., 1998a]. Genetic transmission of the HHV-6 insertion was subsequently suggested in PBMCs of this patient's healthy offspring, a finding that raises significant questions as to the role of high-level latent HHV-6 infection in infectious or

lymphoproliferative disorders [Daibata et al., 1998b]. More detailed molecular studies of other patients are clearly warranted to establish the pathogenetic significance of high-level latent integration of HHV-6 in human disease.

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